PATENT
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In re Application	)	Confirmation No. 3906
Inventor(s): Xianqiang Li et al.	)	Art Unit: 1634
Application No.: 10/053,230	)	Examiner: SISSON, Bradley L.
Filed: January 16, 2002	)	
Title: Method For Isolating And Characterizing Short-Lived Proteins	)	Customer No. 021971

DECLARATION UNDER 37 C.F.R. §1.132

I, Xianqiang Li, declare as follows:

1. I am the co-inventor of the patent application identified above and the subject matters described and claimed therein.

2. I am currently president and CEO of Panomics, Inc., Redwood City, California.

3. From 2000 to present I have been president and CEO of Panomics, Inc. I have conducted nearly 20 years of research and development in the areas of molecular virology, biochemistry, pharmacology, molecular biology, and cellular biology. While in Panomics, I have actively participated in the conception and experimentation of many innovations which have won numerous competitive awards of Small Business Innovation Research (SBIR) by the National Institutes of Health (NIH). The detail of my education and professional experiences is provided in the attached *curriculum vitae* of mine.

4. I have reviewed the Examiner's Office Action mailed April 1, 2004. I believe that the disclosure of our patent application as originally filed adequately describes the claimed invention of a screening assay for short-lived proteins. I also believe that the screening assay is a very useful tool for people in the biotech industry and academia to search for short-lived proteins that are of great biological, diagnostic and therapeutic importance, a utility as asserted by us in the Specification of our application at page 11, lines 1-13. However, to further demonstrate the usefulness of such an assay, I hereby provide supplemental experimental evidence to show that by using the screening assay described in the application, we screened human cDNA libraries and successfully found genes encoding short-lived proteins of biological and therapeutic significance. These assays were conducted either by myself, my co-inventor Dr. Xin Jiang or under our supervision.

5. Construction of a GFP-cDNA Expression Library: Messenger RNAs from human brain, liver, and Hela cell line (Clontech, Palo Alto, CA) were used as templates for cDNA synthesis using a cDNA synthesis kit from Stratagene according to the manufacturer's procedure, with some modifications. The cDNAs were directionally cloned to the C-terminus of EGFP in EGFP-C1/2/3 expression vectors with three open reading frames (Clontech). The expression vectors contain the SV40 origin of replication, which allows easy recovery of cDNA clones that show positive in the screening from cell lines that express the SV40 large T antigen (e.g., 293T).

6. Screening for Mammalian Cells Expressing Diverse GFP Fusion Proteins with Short Half-Lives: The GFP-cDNA expression vector library was transfected into human embryonic kidney 293T cells using Lipofectamine 2000 reagent according to the manufacturer's instructions. The transfected cells were screened by FACS (fluorescence-activated cell sorting) and the cells expressing short-lived proteins were selected based on two criteria: (1) cells that become dimmer after exposure to cycloheximide (CHX), a protein synthesis inhibitor; and (2) cells that become dimmer after a short treatment time, 2 hours. FACS fractionation was used to slice cell population into six subpopulations (R2, R3, R4, R5, R6, R7) of ascending brightness, gating each on successive one-half log intervals of fluorescence. After each subpopulation was divided into two, one subpopulation was treated with 100 ug/ml cycloheximide (CHX) for 2

hours and the other remained untreated. Subpopulations were then re-analyzed to determine whether they had retained a relative distribution consistent with the gating criteria used to obtain this narrow subpopulation and were susceptible to CHX treatment. We found that subpopulations of R3 and R4 ranging from log 2 to log 3 were susceptible to CHX treatment, while R5 and R6 ranging from log 4 to log 5, as well as R7, had no observable response to CHX treatment. The lack of susceptibility of the latter three subpopulations was most likely due to their expressing stable proteins and building up high fluorescence intensity. Subpopulation R4 was further screened for clones expressing short-lived proteins by FACS after 2 hours of treatment with CHX. We found that eight of the 12 groups of cells in subpopulation R4 showed the decrease of the fluorescence intensity by 30 to 50 percent. Individual clones from the cell populations that were responsive to CHX-treatment were further characterized. Clones that showed a decrease in fluorescence intensity ranging from 30 to 90 percent under the treatment of CHX for 2 hours were selected. The cDNAs encoded by the selected 22 clones were sequenced and blasted against the National Center for Biotechnology Information (NCBI) public database. The results are summarized in the table shown below. To the best of our knowledge, there are no published or publicly available sources that provide prior information on whether the proteins we have identified in fact turn over rapidly or not, i.e., are short-lived or not.

Clone	Accession	Gene description	Stability	Estimated half-lives
2	BC005843	Similar to SH3-containing protein	short-lived	2 hours
3	AF176555	A-kinase anchoring protein	short-lived	3 hours
4	AF209502	Calpain	short-lived	1 hours
5	U87277	Splicing factor SRp30c	short-lived	0.5 hour
9	BC000804	Adaptor-related protein complex	short-lived	2 hours
10	BC011384	ATP synthase, H+ transporting	short-lived	2 hours
12	BC005380	Apolipoprotein A-I	short-lived	2 hours
14	BC007513	H19, imprinted maternally expressed	short-lived	2 hours
18	AP000014	Function unknown	short-lived	2 hours
19	M69013	Guanine nucleotide-binding regulatory protein	short-lived	0.5 hour
21	BC003128	CGI-89 protein	short-lived	2 hours
22	AL109795	Function unknown	short-lived	2 hours
23	X89399	Ins(1,3,4,5)P4-binding protein	short-lived	2 hours
25	BC057397	Heat shock 70kD protein 1A	short-lived	2 hours
26	NM_015416	Cervical cancer 1 protooncogene protein p40	short-lived	2 hours
27	AF248272	Gag-pro-pol precursor protein gene.	short-lived	3 hours
28	X07868	Insulin-like growth factors II	short-lived	2 hours
29	NM_000062	Serine (or cysteine) proteinase inhibitor	short-lived	2 hours
30	AF068706	Gamma2-adaptin (G2AD)	short-lived	2 hours
32	AK054590	Function unknown	short-lived	3 hours
33	BC000404	Thyroid hormone receptor interactors 13	short-lived	2 hours

7. Confirmation of Short Half-Lives of Selected Proteins: To directly measure the stability of the selected proteins, three clones were randomly picked and subjected to Western blot analysis. 293T cells were transfected by the clones respectively and treated with or without CHX for 2 hours. The cell lystes were prepared from the cells and the proteins were separated by SDS gel electrophoresis. After transferring to membrane, the short-lived GFP fusion proteins were detected by polyclonal antibodies against GFP tag. All of these proteins degrade in the presence of CHX. However, EGFP protein itself was stable in the same condition. The half-life of each protein determined by Western blot analysis is similar to that determined by analyzing fluorescent decay by FACS, indicating the concurrence between these two analyses. The western blot analysis confirmed the rapid turnover of these proteins that we identified with the screening technology described in the present application.

8. Conclusion: In summary, our experiments demonstrate that by using the screening method described in the present application, a large number of proteins with short intracellular half-lives can be discovered. As listed in the table above, many of the short-lived proteins are of great importance with many practical applications, such as serving as targets for diagnosis and therapeutic intervention of diseases. Examples of such proteins include (e.g., human calpain (clone 4); human apolipoprotein (clone 12); human cervical cancer 1 protooncogene (clone 26); and human insulin-like growth factors II (clone 28). The assay is robust and high throughput because it can be performed without using ubiquitin conjugation as a search criterion. This feature of the invention allows for expanded search for important proteins that are labile but does not require ubiquitin modification for their turnover in the cell. Thus, I believe that the claimed invention is adequately described in the application as originally filed, and is a very useful tool for searching short-lived proteins with important functions in the research and development of disease diagnostics and therapeutics.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the

United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

By: _____

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